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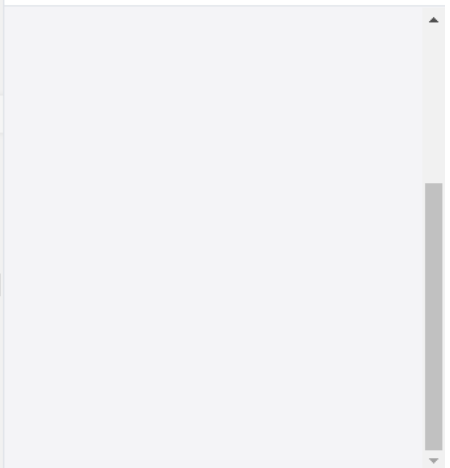
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EFFECT OF HUMAN GRANULOSA CELLS VITRIFICATION ON THE EXPRESSION OF OOCYTE SECRETED FACTORS

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ABSTRACT

Background: oocyte vitrification is a helpful fertility preservation technique for women who are at risk of losing their ovarian functions. Whereas its effect on oocytes has been widely elucidated, the potential effects of this procedure on the biological functions of granulosa cells remain poorly understood. The present study was aimed at assessing the effect of vitrification on the granulosa cells (GCs).

Materials and Methods: a total of 35 women with polycystic ovary syndromes who underwent in vitro fertilization (IVF) in Morula IVF Jakarta Clinic were recruited. The expression of prominent oocytes secreted factors (OSFs) including GDF-9 and BMP-15 were measured at messenger RNA (mRNA) levels. GCs from mature and immature oocytes were collected and measured separately. Relative expression of GDF-9 and BMP-15 was quantified by a real-time quantitative PCR (RT-qPCR) and statistically analyzed using the Mann-Whitney test at a 95% confidence level.

Results: the expression of GDF-9 decreased significantly (0.5-fold, $p < 0.01$) in the immature vitrified GC group but was not in the mature-vitrified GC group. Meanwhile, the expression of BMP-15 was stable in all examined groups ($P > 0.05$).

Conclusion: our results suggest that vitrification may alter oocyte maturation as demonstrated by reduced GDF-9 expression in the immature vitrified GC group.

Keywords: vitrification, granulosa cell, GDF-9, BMP-15, gene expression

INTRODUCTION

Oocyte cryopreservation (OC) has become a fundamental breakthrough for fertility preservation since its first live birth in 1986.¹ Although it was originally intended for women with diminished reproductive potential due to age-related fertility decline, post-surgery decline, or cancer therapy or cancer survivors,² the application of OC is now extending along with the emerging trend of delayed childbearing, postponed marriage, and further diverse indications for ethical, medical, legal, and social bases.^{2,3} Currently, mature metaphase II oocyte vitrification is a gold standard for OC. Women are usually undergoing ovarian stimulation using exogenous gonadotropin for promoting follicular growth and triggering oocyte maturation. After ovum pick-up procedure, maturation assessment is performed by which retrieved mature oocytes are frozen and stored for further use in the future.

Preservation of mature human oocytes through vitrification is well-established and has been widely implemented in IVF laboratories. Vitrification is an ultra-fast amorphous solidification method with a cooling rate of more than 100,000°C/min.⁴ It transforms a living cell into a cryogenic glass-like phase without ice crystal formation thus eliminating potential mechanical injury.^{5,6} A growing body of evidence revealed that vitrification is able to preserve DNA integrity of ovarian follicular cells.⁷ As demonstrated in a study on canine oocytes, it was shown that except for upregulated BCL2 in the vitrified group, the expression of stress-related genes (HSP70 and SOD1), Dnmt1, and BAX genes were comparable between groups.⁸ Moreover, the expression of genes coding for oocyte-secreted factors (GDF9, BMP15, TGFBR1, and BPR2) and apoptosis (BCL2, BAX, and P53) in fresh goat COC were reported equal to the non-vitrified group.⁹

In humans, a comparative study reported that vitrification does not alter biomolecular profiles of oocytes demonstrated by stable post-vitrified expression of TAF4B, GDF9, BMP15,

FIGLA, and POU5f1-OCT4.¹⁰ Furthermore, D'Aurora (2017) revealed that oocyte vitrification does not modify the expression of cytokinesis-related genes essential for chromosome segregation: DCTN1, DCTN2, DCTN3, DCTN6, and PLK1.¹¹

Unfortunately, there is a specific group of cancer patients contraindicated to receive gonadotropin injections such as breast cancer women with estrogen-sensitive type as well as adolescent cancer survivors; thus, retrieving mature oocytes is not possible. As an alternative option, OC through immature oocyte vitrification or ovarian tissue cryopreservation is advisable.¹² On the other hand, success rate of OC through immature oocyte vitrification pertains to some limitations such as low post-warming maturation as well as low fertilization rate. Therefore, the addition of granulosa cells on maturation culture medium for in-vitro maturation improvement is suggested.¹³ Physiologically, GCs are the most vital somatic component of the ovary that privileged bi-directional communication between oocytes and the surrounding cells. Provide physical support and an optimal microenvironment essential for oocyte development and competency, GCs are a vital determinant of oocyte maturity and quality.^{14,15}

While the effect of vitrification on oocytes is studied meticulously, the effect of vitrification on GCs remains paucity. Hence, this study aims to address the largely unexamined effect of vitrification on GC by evaluating the expression of GDF-9 and BMP-15, an oocyte-secreted factor required for oocyte maturation.

MATERIALS AND METHODS

Study design and patient selection

This was an experimental study that took place in Morula IVF Jakarta, Jakarta, Indonesia from July to December 2020. A total of 35 patients who met the following inclusion criteria:

diagnosed with polycystic ovary syndromes (PCOS), aged ≤ 40 years old, underwent first or subsequent cycles, and signed informed consent were recruited to the study. Patients who had endometriosis, adenomyosis, or were identified as poor responders were excluded. PCOS women were determined according to the Rotterdam criteria which were diagnosed by the presence of two out of the three following features: oligo- or an-ovulation, chemical and/or biochemical hyperandrogenism, and polycystic ovarian morphology.¹⁶ Ethical approval was granted by the Ethical Committee of the Faculty of Medicine of the University of Indonesia (KET-995/UN2.F1/ETIK/PPM.00.02/2019). All participants have given their written informed consent.

Collection and isolation of granulosa cells

In order to obtain a complete depiction of the vitrification effect on GCs, samples from mature and immature oocytes were collected separately to avoid possible differences in target gene expression patterns due to different maturation stages of the oocytes. GCs were collected as described previously.¹⁷ After being washed in GMOPS medium (Vitrolife, Sweden), the concentration of GCs in either mature or immature group was estimated using the Neubauer counting chamber in which only samples with GCs $>100,000$ was then proceeded to the next downstream experiment. GCs separation from the extracellular matrix was performed using utilizing density gradients. Histopaque®-1077 medium (Sigma) was utilized by following the manufacturer kit instruction. GCs samples from mature and immature groups were then split and classified into 1) mature fresh group, 2) mature vitrified group, 3) immature fresh group, and 4) immature vitrified group. Fresh GCs sample was diluted in a 500 μL RNA later in a 1.5 mL sterile tube and stored at -20°C for further experiment, while both vitrified mature and immature groups proceeded to vitrification.

Sample vitrification

Prior to the treatment, samples were centrifugated at 300 rcf for 1 minute to separate cells from the buffer solution. The supernatant was then removed and vitrification was initiated by exposing samples to a 50 μ L of vitrification solution 1 (VS1) medium containing 15% ethylene glycol (EG) supplemented with 10% human serum albumin (HSA) for 5 minutes. A subsequent addition of 40 μ L of vitrification solution 2 (VS2, comprised of 15% EG and 15% dimethylsulfoxide (DMSO) supplemented with 20% HSA) was added to the sample tube and left exposed for 30 seconds. The tube was then rapidly plugged in -196°C liquid nitrogen.

Sample warming

The commencement of warming procedure was started with removing the sample from the liquid nitrogen and exposed to 37°C of warm water for 2 minutes. To initiate rehydration, 140 μ L of 0.5M sucrose-concentrated solution was added to the sample tube for 60 seconds and centrifugated at 300 rcf for 1 minute. After removing 100 μ L of supernatant, the sample was loaded into 180 μ L of 0.25 M sucrose solution for 90 seconds. Centrifugation was then repeated (300 rcf, 1 minute) followed by removing 200 μ L of the supernatant. Following the addition of 0.125 M sucrose solution (160 μ L) and exposing it for 60 seconds, the sample was centrifugated. Once the supernatant was removed, the pellet was then resuspended in GMOPS medium for further analysis.

RNA extraction and cDNA synthesis

A high Pure RNA Isolation kit (Qiagen, Germany) was used for total RNA extraction. The procedure followed the manufacturer's instructions. Determination of RNA concentration at 260 nm was conducted. A total of 2 μ g of the total RNA was subsequently transcribed into

cDNA using *ReverTra Ace® qPCR RT Master Mix with gDNA Remover* (Toyobo, Japan). The manufacturer's step-to-step manual was used for cDNA synthesis.

Real-time quantitative PCR (RT-qPCR)

Further analysis for mRNA quantification was employed through RT-qPCR utilizing QuantiTect® SYBR® Green PCR kit (Qiagen, Germany) in combination with the use of human β -Actin (ACTB) as a reference gene for normalization. PCR cycle was conducted in 40 cycles using selected primers (Table 1) with amplification profile as follows: initial denaturation at 95°C for 5 min; denaturation at 95°C for 15 sec, annealing at 57°C for 30 min, and elongation at 72°C for 30 sec. The annealing temperature for BMP-15 was made specific at 59°C for 30 min.

Table 1. Primer design for RT-qPCR

Gene	Primer	Product size/bp	Primer sequences (5'→3')
GDF-9	Forward	199	GGAATCCCAGTCAGGAAGCG
	Reverse		GGCCAAATGAAACCTCGTGC
BMP-15	Forward	196	GGCTCCTAGGGCATTCACTG
	Reverse		CCTCGGTTTGGTCTGAGAGG
ACTB	Forward	80	GTGTGGATTGGTGGCTCTAT
	Reverse		GACTCATCGTACTCCTGCTTG

Data analysis

Baseline characteristics of subjects were presented as number (%) or median-interquartile range for categorical and numerical variables, whichever was most appropriate. All analysis was performed using the Statistical Package for the Social Sciences (SPSS). 95% confidence level was set.

RESULTS

The baseline characteristics of subject participants were demonstrated in Table 2. As shown in the table, the mean age of subjects was 32 years old with the mean duration of infertility was six years. Among all participants, 29 (93.5%) women had primary infertility, while 2 (6.5%) women are identified to have secondary infertility. The mean BMI, anti-Mullerian hormone (AMH) level and antral follicle count (AFC) were 24.04 kg/m², 3.43 ng/mL, and 15 follicles, respectively. The basal FSH, LH, estradiol, and progesterone were at the normal level.

Table 2. Baseline characteristics of the studied population

Characteristics	Mean ± SD
	Median (Min-Max) n (%)
Age (years)	32,39 ± 3,33
Duration of infertility	6 (0,6–13)
Type of infertility n (%)	
Primary	29 (93,5)
Secondary	2 (6,5)
BMI (kg/m ²)	24,04 ± 4,73
AMH (ng/mL)	3,43 (1,58–13,50)
AFC	15,26 ± 6,92
FSH basal (mIU/mL)	5,96 ± 1,14
LH basal (mIU/mL)	6,25 ± 2,27
Estradiol basal (pg/mL)	31,89 ± 12,04
Progesterone basal (ng/mL)	0,16 ± 0,08

Data are presented as the number of subjects and percentage [n (%)] and as median (min-max)

In terms of gene expression, GDF-9 expression was reduced significantly by 0.5-fold in the immature vitrified group (P=0.01, Figure 1). PCR result analysis showed that in the mature vitrified group, the expression of GDF-9 was slightly decreased but it was not statistically

significant (Figure 1). Meanwhile, the abundance of BMP-15 was comparable in all examined groups (Figure 1).

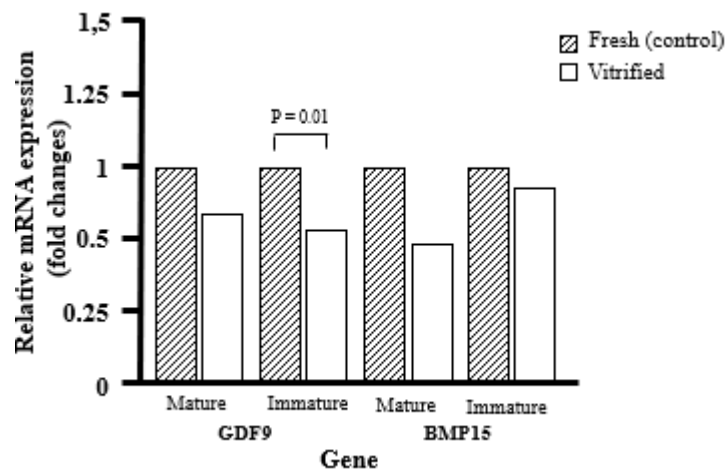


Figure 1. Relative mRNA expression of GDF-9 and BMP-15. The amount of target genes mRNA is normalized to the amount of ACTB mRNA. All values are presented as mean \pm standard error (SEM) and were analyzed by a Mann-Whitney test at a 95% confidence level.

DISCUSSION

The present study observed that the expression of GDF-9 was reduced significantly after vitrification in immature GCs group in comparison to immature fresh group. Conversely, post-vitrification of mature GCs group did not differ compared with fresh mature group signifying less void of vitrification impact on mature GCs. Moreover, the expression of BMP-15 was unaltered in all groups. Our study has addressed the important issue of assessing the effect of vitrification separately on mature and immature GCs.

This present finding was partially in concordance with the previous report which observed compromised GDF-9 and BMP-15 expression on bovine cumulus-oocyte complex (COC) after vitrification.¹⁸ Supporting the previous result, it was suggested that vitrification did not influence the genetic profiles of GDF-9 and BMP-15 in ovarian tissue.¹⁹ The discrepancy between our results from others could be attributed to the different nature of the samples for

analysis. Our study solely used GCs without including the oocytes while most studies have investigated the effect of vitrification on COC and ovarian tissue.

GDF-9 is a vital oocyte-secreted factor member of the TGF- β superfamily that plays a critical role in cumulus cell proliferation, expansion, and the subsequent development of COCs,²⁰ and also for supporting oocyte maturation process.²¹ In vitro study showed that GDF-9 supplementation promoted the transition of primary follicles resulting in a decreased number of primary and secondary follicles and an increase in pre-antral and antral follicles suggesting that larger follicles were only stimulated to advance to later developmental stages by the presence of GDF-9.²² A study on animal models furtherly demonstrated that GDF-9 is mainly expressed in the early developmental stages, with the highest and lowest expression observed in germinal vesicles and mature oocytes, respectively.²³ The finding of altered expression of GDF-9 after vitrification in immature GCs group in the present study seems to explain the underlying cause of low post-warming maturation and fertilization; thus limiting the clinical use of immature oocyte vitrification. The yield in this investigation suggests previous practical idea that supplementation of post-vitrified immature oocytes with GDF-9 synthetic might be necessary to promote in-vitro maturation.²¹

The establishment of vitrification procedure is reflected in the current literature. Although employing slightly different types of cryoprotectants with different concentrations,^{24,25} a consistent remarkably good quality preservation through vitrification method is apparent. Our result corroborates previous results which suggest that vitrification, as well as cryoprotective agents, proficiently preserves organelles, cells, tissues, and any other corresponding cellular processes which in turn, explains an unaltered expression of GDF-9 and BMP-15 in this present study except for GDF-9 expression in the immature vitrified group.

CONCLUSION

In conclusion, our study suggests that vitrification may alter oocyte maturation by reduced GDF-9 expression in immature GCs.

Declaration

Availability of data and materials

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interest

The authors have no conflicts of interest to declare.

Funding

The authors receive no specific grant to fund the study

Authors' contribution

BS contributed to the study's conception and design. BW, RM, and AB performed methodology development, study validation, and supervision. BS contributed to the data analysis, performed experiments, and wrote the original draft. All authors conducted data validation, visualization, and critically revised the manuscript. All authors read and approved the final manuscript.

Acknowledgment

Not applicable.

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Contribution Details (to be ticked marked as applicable):

	Batara Sirait	Budi Wiweko	R. Muharam	Ahmad Aulia J	I.A. Rachman	Arief Boediono
Concepts	√	√				√
Design	√	√				√
Definition of intellectual content			√	√	√	
Literature search	√					
Clinical studies	√	√	√	√	√	√
Experimental studies	√					
Data acquisition	√					
Data analysis	√					
Statistical analysis	√					
Manuscript preparation	√					
Manuscript editing	√	√	√	√	√	√
Manuscript review		√	√	√	√	√
Guarantor		√				√

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Instructions

The purpose of this form is to provide readers of your manuscript with information about your other interests that could influence how they receive and understand your work. The form is designed to be completed electronically and stored electronically. It contains programming that allows appropriate data display. Each author should submit a separate form and is responsible for the accuracy and completeness of the submitted information. The form is in six parts.

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This section asks for information about the work that you have submitted for publication. The time frame for this reporting is that of the work itself, from the initial conception and planning to the present. The requested information is about resources that you received, either directly or indirectly (via your institution), to enable you to complete the work. Checking "No" means that you did the work without receiving any financial support from any third party -- that is, the work was supported by funds from the same institution that pays your salary and that institution did not receive third-party funds with which to pay you. If you or your institution received funds from a third party to support the work, such as a government granting agency, charitable foundation or commercial sponsor, check "Yes".

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Batara

2. Surname (Last Name)

Sirait

3. Date

22/06/2023

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Yes No

5. Manuscript Title

EFFECT OF HUMAN GRANULOSA CELLS VITRIFICATION ON THE EXPRESSION OF OOCYTE SECRETED FACTORS

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Letter of Acceptance
27 June 2023

**Dear: Batara Sirait^{1,2,3,4}, Budi Wiweko^{5,6}, R. Muharram⁶, Ahmad Aulia Jusuf⁷,
Ichramsjah A. Rachman⁶, Arief Boediono^{3,4,8}**

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I am very excited to accept your paper entitled:

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Your paper will be published in the issue of Vol. 12 Number 2, 2023.

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Please do not hesitate to contact us if you need anything. It has been a pleasure for us to proofread and edit your work, and we are looking forward to your colleagues and your other papers in the near future.

Agreed/Menyetujui by:

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